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Lymphopenia in occupational pulmonary silicosis with or without autoimmune disease

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SUMMARY

An increased prevalence of autoimmune diseases such as rheumatoid arthritis has been demonstrated in silica-exposed patients. The aim of this study was to determine the peripheral blood lymphocyte phenotype in a population of silicotic workers employed in the slate mines of the district. Silicosis was assessed in 58 patients according to the International Labor Office's criteria. Clinical and biological data including flow cytometric evaluation of the lymphocyte subsets were compared with those from 41 healthy volunteers. The silicotic patients had a higher prevalence of autoimmune diseases (6/58 *versus* 0/41: $P < 0{\cdot}05$) and of elevated antinuclear antibody titres compared to the control group. A very significant decrease of total lymphocyte count ($P < 0{\cdot}001$) involving B, T and Natural Killer cells was found in silicotic patients as compared with matched healthy volunteers. A significant increase in the percentage of activated T cells (12·3%) was observed in the silicotic group as compared to 6·5% in the control group ($P = 5 \times 10^{-5}$). Our results show that in silicotic patients, the absolute number of circulating lymphocytes is diminished with an increased proportion of activated T cells. Whether these findings could predispose to the development of autoimmune disorders is discussed.

Keywords pulmonary silicosis autoimmunity lymphopenia activated T cells

INTRODUCTION

An increased prevalence of autoimmune diseases (AD) such as rheumatoid arthritis [1] (RA) or systemic sclerosis [2] has been demonstrated in silica-exposed patients from the beginning of the century. Anti neutrophil cytoplasmic antibodies (ANCA) associated diseases have been reported [3] and silica dust exposure was reported by 46% of patients with ANCA-associated small vessel vasculitis compared with 20% of control subjects [4]. We reported on ANCA-associated glomerulonephritis in former slate workers [5].

The pathophysiology of pulmonary silicosis is wellknown. Following inhalation, silica particles enter the lung and are subsequently found in the alveolar space, the lung tissue and the pulmonary lymphatics. Alveolar and interstitial macrophages are activated after particle uptake; they produce reactive oxygen species, lysosomal agents, pro-inflammatory or pro-fibrotic cytokines such as IL-1, TNF-α, TGF-β, PDGF and products of arachidonic acid metabolism [6]. The recycling of the silica particles by other macrophages, the recruitment of various inflammatory cells, the development of pulmonary fibrosis and of granulomas with profound alteration of lung structure are the following steps leading to the pulmonary disease. Moreover silica particles can be shown in tiny quantities in the blood stream [7], and extrapulmonary lesions have been described with silica nodules present in liver, spleen, extra thoracic lymph nodes and bone marrow [8]. In contrast with the function of macrophages in the course of the disease, little is known about the role of lymphocytes in silicosis or about the mechanisms leading to the development of autoimmune disorders. Macrophages, B and T cell subsets have been described in the lung and bronchoalveolar lavage fluid after acute exposure [9] but very few data are available in blood [10].

So we have studied a cohort of assessed silicotic men in steady state in order to determine the prevalence of clinical and immunological disorders and to look for differences in lymphocyte subsets in blood when compared with non-exposed controls.

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METHODS

Population selection

Patients (group 1) were recruited inside a cohort of 114 silicotic slate miners registered for their degree of disability related to pulmonary silicosis at the Union Régionale de l'Ouest, Sécurité Sociale Minière on January 1995. We sent them by mail a

proposal for entering a medical investigation dealing with silicosis and autoimmune diseases. Individuals who accepted to join the study sent their personal agreement for attending a medical consultation in the department of nephrology at the Angers Hospital University. They gave a written agreement after a talk with the consultant before starting the examination.

The control group (group 2) was composed of volunteer men without any history of professional silica exposure (clergymen, third age university students, retired military and retired teachers). The same mailing procedure was used to get in touch with them, and their agreement was obtained in the same way. Men over 77 years or presenting acute evoluting diseases were excluded from the study. Our hospital ethic committee gave consent to the study.

Silicosis was assessed according to the International Labor Office criteria: combination of long-lasting (at least 5 years) exposure to silica in slate mining with characteristic abnormalities on chest X-ray. A three physician college assessed the percentage of the impairment related to silicosis.

Medical examinations with specific search for overt AD were carried out by the same physician (JFS) for all patients in the morning, and afterwards urine and blood samples were collected. The prevalence of smoking, of urogenital and cardiovascular diseases was identical in both groups. In the same way, the prevalence of medications other than immunosuppressives was similar in the two groups. The mean age of the control group (67 years $\pm$ 4·5) was significantly lower when compared to that of the silicotic group (70 years $\pm$ 3·5). Body mass index was within the normal range and did not differ between the two groups.

Laboratory tests

The following tests were performed: creatininaemia, creatininuria, proteinuria. No significant difference was observed between the two groups.

Rheumatoid Factors (RF), C3 and C4 complement components were measured in serum by nephelometry (Dade Behring) and serum protein immunoelectrophoresis was achieved.

The plasmatic interleukin-1 beta (IL-1β) was measured by the ELISA TiterZyme IL-1β EIA Kit (Bio Advance Emerainville France). Circulating angiotensin converting enzyme (ACE) was quantified by spectrophotometry (Sigma diagnostic), normal values are: 30 $\pm$ 11 U/l. Antinuclear antibodies (ANA) were detected by indirect immunofluorescence (IIF) on HEp2 cells, starting the screening at a dilution of 1/100, and antidouble strand anti-DNA antibodies were detected by IIF on *Crithidia luciliae*. ANCA were detected by IIF on ethanol fixed normal human neutrophils, antimyeloperoxidase (MPO) and antiproteinase 3 (PR-3) antibodies by ELISA (Euroimmun Gross Groenau, Germany).

Lymphocyte phenotyping

The number of lymphocytes was calculated using a Coulter MAXM haematology analyser. Peripheral blood lymphocyte subsets were assessed by two or three colour flow cytometry analysis with a FACScan (Becton Dickinson, San José, CA). CellQuest software was used for the flow cytometric data acquisition and data analysis. FITC-, PerCP- or PE-conjugated monoclonal antibodies against CD3, CD5, CD11b, CD14, CD16, CD19, CD25, CD45, CD56, HLA DR (Becton Dickinson, San José, CA), CD4, CD8 (Becman Coulter, Miami, FL), and the relevant isotype controls were used.

Quality control for immunophenotyping was performed by verifying internal consistency for $CD3^+$ lymphocytes present in each reaction tube containing the CD3 monoclonal antibody. The sum of $CD3^+$, $CD19^+$ and $CD16/CD56^+$ percentages was approximately 100 $\pm$ 5% for each sample analysed.

Statistical analysis

Data were expressed as mean $\pm$ standard deviation. Statistical comparisons between mean values were carried out using the Student's *t*-test and Mann–Whitney's *U*-test when necessary. χ^2 with Yate's correction and Fisher's test were used for the comparisons of the percentages between the two groups. All tests were realized using SPSS.9.0 package.

RESULTS

Subjects

Over the 114 silicotic slate miners registered, 23 deceased during the two years of time separating the registration date and the start of the survey, 22 refused to be involved in the study and 11 didn't answer. Fifty-eight men (group 1) entered the study. The control group included 41 men who accepted to participate in the study under the same conditions as silicotic subjects (group 2).

The prevalence of AD history was significantly higher in group 1 as compared to group 2: 11% *versus* 0% ($P < 0{\cdot}05$). As shown in Table 1, six patients exhibited some form of AD; 52 subjects were AD free. Three patients (patient nos 2, 3 and 4) had an active seropositive RA under treatment, one (patient no. 1) suffered a documented sensory neuropathy of unknown origin and RA in remission at the time of the study. The fifth patient had biopsy proven pauci immune glomerulonephritis. The sixth patient, who had myasthenia gravis with circulating antilaminin, antismooth and antistriated muscle antibodies but no antiacetylcholine antibodies, had manifested clinical improvement under prolonged prostigmin therapy. These two latter patients were treated with steroids and the three patients suffering from active RA were treated with gold salts plus steroids (patients no. 3 and no. 4) and anti-malarial drug (patient no. 2). All six patients had high ANA titre, but no ANCA were detected.

Immunological data

As shown in Table 2, the levels of mean plasmatic IL-1β and

Table 1. Patients with medical history of autoimmune disease

	% sil	Diagnosis	RF	ANA	ANCA	Treatment
Patient no. 1	20	RA + SN	3	1/200	Neg	0
Patient no. 2	10	RA	216	1/200	Neg	Anti malarial
Patient no. 3	50	RA	28	1/200	Neg	Gold/steroids
Patient no. 4	15	RA	750	1/5000	Neg	Gold/steroids
Patient no. 5	20	GN	3	1/2000	Neg	Steroids
Patient no. 6	35	MG	3	1/1000	Neg	Steroids

% sil, percentage of impairment related to pulmonary silicosis; RA, rheumatoid arthritis; GN, glomerulonephritis (pauci imune GN); MG, myasthenia gravis; RF, rheumatoid factor: nl < 15 UI/ml; SN, sensory neuropathy; gold, gold salt treatment.

Table 2. Biological data

	Group 1 $n = 58$	Group 2 $n = 41$	P value Group1/group2
IL-1β (pg/ml)	145 ± 73	110 ± 50	0·011*
ACE (U/l n: 5–33)	24·5 ± 14	17 ± 6·5	0·002*
ANA ≥ 1/200	28/58	4/41	$3{\cdot}4 \times 10^{-4}$†
ANCA	3/58	0/41	ns‡
RF (< 15; 15–30; ≥ 30)	42; 9; 6	33; 4; 4	ns†
C3 (mg/dl)	93 ± 22	86 ± 15	ns§
C4 (mg/dl)	34 ± 13	34 ± 12	ns§

n, number of subjects; IL-1β, interleukin 1 beta; ACE, angiotensin converting enzyme; ANCA, anti-neutrophil cytoplasmic antibody.

ANA results are expressed in number of patients with ANA titre ≥ 1/200.

RF, rheumatoid factor. Results are expressed in number of patients with normal (< 15 IU/ml), slightly increased (15–30 IU/ml) and markedly increased (≥ 30 IU/ml).

C3: nl: 50–105 mg/dl; C4: nl: 15–45 mg/dl.

Statistical comparisons are between groups 1 and 2.

*Mann–Whitney's *U*-test.

†χ^2 test.

‡Exact Fisher's test.

§Student's *t*-test.

ACE were significantly higher in group 1 than in group 2 as already described [11,12]. The other immunological parameters did not differ between both groups except for ANA, which were significantly higher in group 1 as compared to group 2; a speckled aspect in IIF was observed in all individuals except for two who exhibited either an homogenous aspect or a speckled and a nucleolar pattern. The *Crithidia luciliae* test was negative in all patients. Three subjects in group 1 had positive ANCA (p-ANCA with anti-MPO specificity, c-ANCA with uncertain antigenic specificity and atypical ANCA). All three positive subjects were free of any clinical or biological symptoms of systemic vasculitis. Five subjects had a monoclonal gammapathy in group 1 *versus* one in group 2, the difference was not significant ($P = 0{\cdot}2$).

Lymphocyte phenotyping

Complete data were available for all individuals in the control group and for 53 of 58 in group 1. All subjects had normal haemoglobin concentration as well as neutrophil and platelet count. As shown in Table 3, the major finding was a very significant decrease in the absolute number of circulating lymphocytes in group 1 (1256×10^3/ml) as compared to group 2 (1856×10^3/ml). This decrease involved $CD3^+$ (T), $CD19^+$ (B), $CD16^+CD56^+$ (NK), $CD4^+$, $CD8^+$ and $CD25^+$ cells but not $CD3^+$ HLA DR^+ (activated T) cells. These abnormalities were observed in all but five silicotic individuals. The $CD4^+/CD8^+$ ratio was normal in both groups. The percentages of the different lymphocyte subsets did not differ between both groups except for the percentage of $CD3^+$ HLA DR^+ (activated T cells) that was significantly higher in group 1 as compared to group 2. No significant difference was observed for any parameter within group 1 between those with or without AD.

DISCUSSION

These clinical data are in agreement with those previously reported by others although none of our patients had either systemic sclerosis, lupus syndrome or systemic vasculitis, but the prevalence of these AD in the silicotic population is very low (about 0·2%) [13]. The biological and immunological abnormalities observed in our population have also been previously described [11,12,14–17]. In a retrospective study (unpublished data) we found a 13% (11/85) prevalence of professional silica dust exposure in men over 40 years admitted in our ward for primary glomerulopathy compared to a 2·3% prevalence in the general population of the district ($P = 4{\cdot}3 \times 10^{-6,}$ χ^2 test). Circulating ANCA with antimyeloperoxidase specificity were detected in five of 10 of these patients. These data are in agreement with the results of Hogan *et al*. in a study conducted in the south-east of United States [4].

The major finding that emerge from this study is the very significant decrease in the number of the different lymphocyte subsets and the increased ratio of activated T cells in the blood. To the best of our knowledge this has not been reported before. Only Watanabe *et al*. (1987) [10] reported on the occurrence of a

Table 3. Blood cell counts and cell subsets percentage

	Group 1 ($n = 53$)	Group 2 ($n = 41$)	P value
Lymphocytes	**1256 ± 443**	**1856 ± 578**	$\mathbf{8 \times 10^{-7}}$*
T cells	**880 ± 369** (68·5%)	**1301 ± 490** (69·5%)	$\mathbf{4 \times 10^{-5}}$*
B cells	**116 ± 82** (9·3%)	**180 ± 139** (9·6%)	$\mathbf{1 \times 10^{-6}}$*
NK cells	**210 ± 114** (17%)	**321 ± 168** (18%)	**0·001**†
$CD4^+$ cells	**506 ± 219** (39%)	**756 ± 229** (42%)	$\mathbf{4 \times 10^{-6}}$*
$CD8^+$ cells	**448 ± 220** (35%)	**642 ± 359** (33%)	**0·005***
($CD3^+$ DR^+) cells	106 ± 86 (**12%**)	87 ± 56 (**6·5%**)	$\mathbf{2{\cdot}3 \times 10^{-5}}$‡
$CD25^+$	**46 ± 26** (3·9%)	**65 ± 43** (3·6%)	**0·03**†

n, number of subjects.

Cell count is expressed in mean number $\times 10^3$/ml ± standard deviation. Percentages of lymphocyte subsets ± standard deviation.

Bold type indicates data with significant differences between group 1 and group 2.

*Student's *t*-test.

†Mann–Whitney's *U*-test.

‡χ^2 test.

significant decrease in peripheral $OKT3^+$ cells count. Struhar *et al.* (1989) [9] and Sanchez-Roman *et al.* (1993) [18] did not report on lymphopenia in their studies.

These results raise two important questions concerning the mechanism of lymphopenia and its potential role in the occurrence of AD. Malnutrition is an unlikely hypothesis for lymphopenia since the body mass index of the two groups was equivalent; the difference in the mean age between the two groups (3 years) is too weak to explain the difference observed [19].

The increased percentage of activated T cells could reflect a chronic stimulation of T cells by silica particles liberated from damaged macrophages [8]. Interestingly it has been shown [20] that silica may act as a superantigen and that lymphocytes incubated with silicate became apoptotic through the Fas/Fas ligand pathway [21]. Moreover serum levels of soluble Fas ligand are elevated [22] and an overexpression of soluble Fas mRNA [23] have been observed in peripheral blood mononuclear cells of silicotic individuals. Apoptosis could very well explain the T cell lymphopenia observed in this study; however, it does not explain the decrease in B and NK cell counts. Lymphocyte sequestration in enlarged silicotic nodules [8] could be another potential mechanism of lymphopenia.

The other point to be discussed concerns the potential role of lymphopenia in the occurrence of autoimmunity. Several experimental data now strongly support the role of lymphopenia in autoimmune diseases. Lymphopenia, whether congenital in BB rats or experimentally induced by irradiation, thymectomy or cyclophosphamide, is commonly associated with the onset of autoimmune disorders (review in [24]). Furthermore, in some experimental models, the disease can be prevented by reconstitution with cells of a specific phenotype [25]. The importance of the $CD4^+$ $CD25^+$ subset in the maintenance of immunological self tolerance is crucial [26] since elimination of $CD25^+$ T cells in normal mice leads to the development of autoimmune diseases. It is interesting that in our population the absolute number of $CD25^+$ cells was diminished to the same extent as the other lymphocyte subsets.

In coeliac disease, Di Sabatino [27] showed a decrease in the absolute number of $CD3^+$, $CD4^+$, $CD8^+$ and $CD19^+$ cells in untreated patients as compared with treated patients and healthy volunteers. The proportion of $CD3^+$ HLA DR^+ cells was found higher in untreated patients than in controls (12·8% ± 7 *versus* 6·2% ± 3). Furthermore, in systemic lupus erythematosus and primary vasculitides, a decrease in total lymphocyte count related to activation induced cell death has been demonstrated *in vitro* [28]. These data are reminiscent of our own.

It is noteworthy that lymphopenia was observed in 48/53 of silicotic subjects while only 10% develop overt clinical autoimmune disorders. This indicates that other genetic (MHC?) or environmental factors are required for the development of autoimmune diseases.

In conclusion, our results show that in men with occupational pulmonary silicosis the absolute number of circulating T, B and NK cells was dramatically reduced when compared to healthy volunteers. The percentage of each lymphocyte subset was identical in the two groups except for the percentage of activated T cells that was significantly increased in the silicotic group. The presence of lymphopenia and relative increase in circulating activated T cells could be part of different factors that initiate the onset of autoimmune disorders in this population.

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